[SPECIFICATION]

**[TITLE OF THE INVENTION]**

Method for Producing Embryonic Germ Cell Line in Aves

**[BRIEF DESCRIPTION OF THE DRAWINGS]**

Fig. 1 is a photograph under a stereomicroscope showing chicken embryonic germ cell colonies after 3 passages on chicken embryonic fibroblasts as a feeder cell.

**[DETAILED DESCRIPTION OF THE INVENTION]**

**[OBJECTS OF THE INVENTION]**

**[FIELD OF THE INVENTION AND PRIOR ARTS]**

The present invention relates to a process for preparing an established avian embryonic germ cell line. More specifically, the present invention relates to a process for preparing an established avian embryonic germ cell line, comprising subculturing primordial germ cells isolated from avian embryo in a medium containing a suitable cell growth factor, differentiation inhibitory factor and feeder cells.

Embryonic stem (ES) cells are undifferentiated and pluripotent cells isolated from blastocyst or morula embryos. To date, ES cells derived from mouse (Evans, M. J. and Kaufman, M. H., *Nature*, 292:154-156 (1981); and Bradley et al., *Nature*, 309:255-256 (1984)), bovine (First, N. L. et al., *Reprod. Feril. Dev.*, 6:553-562 (1994)), porcine (Wheeler, M. B., *Reprod. Fertil. Dev.*, 6:563-568

(1994)), sheep (Piedrahita, J.A. et al., *Theriogenology*, 34:879-901(1990)), rabbit (Giles, J. R. et al., *Mol. Reprod. Dev.*, 36:130-138 (1993)) and rat (Iannaccone, P.M. et al., Dev.Biol., 163:288-292(1994)) have been characterized. Recently, there is a report relating human ES cells (Thomson, J.A. et al., Science, 282:1145-1147(1998)). ES cells are expected to be highly useful, e.g., in the study of developmental biology, analysis of the characteristics of totipotent cells, and gene-manipulation to produce genetically modified livestock. However, only mouse ES cells with proven germ-line transmission have been established (Bradley et al., *Nature*, 309:255-256 (1984)), and the use of ES cells in producing livestock has not yet become a reality because of limitations in germ-line transmission in species other than mice.

Recently, primordial germ cells (PGCs) have been provided as an alternative source of pluripotent stem cells (Resnick, J. L. et al., *Nature*, 359:550-551 (1992)). PGCs, the progenitors of sperm or egg cells that develop after sexual maturity, are originated from mesoderm in mice and migrated to gonad at early stage of embryo development (Ginsburg, M. et al., *Development*, 110:521-528(1990)). Mouse PGCs have been successfully co-cultured by Godin et al. on mitotically inactivated STO cells supplemented with three critical growth factors: stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor(bFGF) (Godin, I. et al., *Nature*,

352:807-809(1991); Dolci, S. et al., *Nature*, 352:809-811 (1991); Matsui, Y. etal., *Nature*, 353:750-752 (1991); and Resnick, J. L. et al., *Nature*, 359:550-551 (1992)). Resnick et al. firstly reported that mouse PGCs are successfully subcultured and called as embryonic germ cell line (EG cell line) (Resnick, J. L. etal., *Nature*, 359:550-551 (1992)). In addition, Labosky et al. demonstrated that murine EG cell lines permitted to produce germline chimera (Labosky, P. A. et al., *Development*, 120:3197-3204 (1994)). However, hitherto, other animals than mouse have been reported in the production of germline chimera and particularly, other species than mammals have been reported in the establishment of ES cell line, while as to bovine (Cherny, R. A. et al., *Reprod. Fertil. Dev.*, 6:569-575 (1994)) and porcine EG cells (Shim, H. et al., *Biol. Reprod.*, 57:1189-1095 (1997); Piedrahita, J. A. et al., *J. Reprod. Fertil.(suppl)*, 52:245-254 (1997)), there have been characterized traits such as morphology, alkaline phosphatase activity, and embryoid body formation.

Avian PGCs exhibit other migration pattern than that of mammals. In avian species, PGCs first arise from the epiblast and migrate to the hypoblast of the area pellucida (the germinal crescent) at stage 4, approximately 18-19 hours after incubation (Eyal-Giladi, H. and Kochav, S., Dev. Biol., 49:321-337 (1976); Swift, C. H., Am. J. Anat., 15:483-516 (1914); Hamburger, V. and Hamilton, H. L., J. Morphol., 88:49-92(1951)). PGCs move

from the germinal crescent into the blood stream at stage 10-12 (Ando, Y. and Fujimoto, T., Dev.Growh Differ., 25:345-32 (1983); and Ukeshima, A. et al., J. Electron. Microsc., 40:124-128 (1991)) and circulate in the vascular system until stage 17 (2.5 days of incubation) when they reach the region of the germinal ridges, in which they finally concentrate and colonize (Nieuwkoop, P. D. and Sutasurya, L. A., In Primordial Germ Cells in the Chrodates, 113-127 (1979)).

Allioli et al. reported that chicken PGCs isolated from gonads could proliferate for several days under an in vitro culture condition (Allioli, N. et al., Dev. Biol., 165:30-37 (1994)). Chang et al. cultured chicken PGCs from gonads on stroma cells of the germinal ridge for 5 days and verified that these cultured gonadal PGCs had the ability to migrate to the germinal ridge when re-injected into recipient embryos (Chang, I. et al., Cell Biol. Int., 19:569-676(1995 b)). Recently, Chang et al. produced germ-line chimeric chickens by injection of gonadal PGCs which had been cultured in vitro for 5 days (Chang, I. et al., Cell Biol. Int., 21,495499 (1997)). However, in order to produce transgenic avian through transfection and gene-targeting, the cell line permitting the germline transmission of foreign gene injected is required, so that there is a need in the art for a process for preparing an established avian EG cell line.

[TECHNICAL PROBLEMS TO BE SOLVED BY THE INVENTION]

The present inventors have made intensive researches to establish avian embryonic germ cell line to proliferate stably and continuously, and as a result, succeeded in the establishment of pluripotent embryonic germ cell by subculturing gonadal primordial germ cells isolated from chicken embryo under suitable conditions.

Accordingly, it is a primary object of the present invention to provide a process for establishing an avian embryonic germ cell line.

[ELEMENTS AND OPERATIONS OF THE INVENTION]

The present process for preparing an avian embryonic germ cell line will be described in more detail as follows:

The embryo of White Leghorn at 5.5 days was treated with trypsin-EDTA to obtain a mixed cell suspension containing gonadal primordial germ cells (gPGCs). The mixed cell suspension obtained in Example 1 was added to DMEM supplemented with chicken serum, a cell growth factor including SCF (stem cell factor), bFGF (basic fibroblast growth factor), IL-11 (interleukin-11) and IGF-I (insulin-like growth factor-I) and LIF (leukemia inhibitory factor) to inhibit cell differentiation and incubated in an incubator at 37℃ under an atmosphere of 5% $CO_2$ to produce colonies. After the incubation for 7-10 days, the colonies

of PGCs were observed on a layer of germinal ridge stroma cells (GRSC). The colonies of PGCs were separated from the GRSC layer by gentle pipetting and harvested by centrifuge. The harvested cells were resuspended in a fresh medium, divided into a fresh plate together with chicken embryonic fibroblasts (CEFs). After the incubation for 7-10 days, the colonies of EG cells were formed. The EG cell line obtained was maintained for up to 10 passages and proliferated over a period of 16 weeks in repeated subculture.

The present process for preparing EG cell line is applicable to a variety of birds including turkey, quail, pheasant and duck as well as chicken such as White Leghorn. PGCs useful in this invention are derived from embryonic blood and germinal crescent as well as embryonic gonad. The embryo as PGC source is not restricted with respect to the stage of embryo development unless the embryo inhibits the accomplishment of the purpose of this invention. The embryo at a suitable stage varies depending on avian species and organ from which PGCs are isolated.

The following Examples are intended to further illustrate the present invention without limiting its scope.

EXAMPLE 1: Isolation and Culturing of PGCs

A fertilized egg of White Leghorn (WL) obtained from

the College of Agriculture and Life Sciences, Seoul National University was incubated for 5.5 days (until stage 28) at 37.5 C and a relative humidity of 60-70%.

The embryo was extracted from the fertilized egg incubated for 5.5 days and washed in a 100 mm petri dish with magnesium-free phosphate buffered saline (PBS) to remove the yolk and blood. The embryo was transferred to a petri dish coated with a black wax and the embryonic gonads were isolated therefrom with forceps. The gonad tissue was separated into individual gonadal primordial germ cells by treating with 0.25% trypsin-0.05% EDTA. Added thereto was DMEM (Dulbecco's Modified Eagle's Medium, Gibco BRL, USA) containing 10% FBS (fetal bovine serum, Gibco, USA) to inactivate trypsin, and the mixed cell suspension containing gonadal primordial germ cells (gPGCs) were harvested.

Thereafter, to establish the conditions for culturing EG cells, the following experiments were performed. The mixed cell suspension was diluted with DMEM (Gibco BRL, USA) containing interleukin-11 (IL-11) and insulin-like growth factor-I (IGF-I) as well as stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF), which have been reported to be important growth factors for the survival and proliferation of PGCs in the mouse. IL-11 has been suggested to accelerate the growth of mouse PGCs cultured in vitro and influence on the growth of chicken embryonic cell (Koshimizu et al., Development, 122:1235-1242(1996);

Pain et al., Development, 122:2339-2348(1996)). IGF-I has been reported to be involved in the proliferation of chicken PGCs (Chang et al., Cell. Biol. Int., 19:143-149(1995a)). The gPGCs were then incubated at 37℃ under an atmosphere containing 5% $CO_2$ until the colonization of the PGCs. Since tests showed that the colonization of PGCs does not occur in the absence of IL-11 and IGF-I, IL-11 and IGF-I as well as SCF, LIF and bFGF are considered to be essential for the prolonged survival, proliferation and colonization of chicken gPGCs.

EXAMPLE 2: Establishment of Embryonic Germ Cell Line

The mixed cell suspension obtained in Example 1 was diluted to a suitable concentration with DMEM (Gibco, USA) supplemented with 10% FBS, 2% chicken serum, 1mM sodium pyruvate, 2mM L-glutamine, 5.5 x $10^{-5}$M 2-mercaptoethanol, 100 μg/ml of streptomycin, 100 units/ml of penicillin, 5 ng/ml of human stem cell factor (hSCF), 10 units/ml of murine leukemia inhibitory factor (mLIF), 10 ng/ml of bovine basic fibroblast growth factor (bFGF), 0.04 ng/ml of human interleukin-11 (h-IL-11) and 10 ng/ml of human insulin-like growth factor-I(IGF-I). Then, the suspension was incubated in an incubator for 7-10 days at 37℃ under an atmosphere of 5% $CO_2$ to produce PGC colonies. After the incubation for 7-10 days, the colonies of PGCs were observed on a layer of germinal ridge stroma cells (GRSC). The colonies of PGCs were separated from the GRSC layer by gentle pipetting and harvested by centrifuge at 200 X g

for 5 min. The harvested cells were resuspended in the same medium, divided into a fresh 24-well plate together with chicken embryonic fibroblasts (CEFs), which were not mitotically inactivated, and then cultured at 37℃ under an atmosphere of 5% $CO_2$ until the colonies of EG cells were generated. In a preliminary experiment, it was revealed that the proliferation of EG cells was negligible when STO cells or CEF treated with mitomycin to become mitotically inactive were used as a feeder cell. Therefore, the present invention employs CEF not subject to be mitotically inactive as a feeder cell for subculturing EG cells. The subsequent subculturing was performed at an interval of 7 to 10 days under the condition as above. The EG cell line was maintained for up to 10 passages and proliferated over a period of 16 weeks in repeated subculture.

The EG cell line established above was observed under a stereomicroscope. Even though the morphology of the chicken EG cell line was slightly different from that of mouse ES cell line or EG cell line, the whole morphology of colonies were similar to that of mouse or porcine ES cell line or EG cell line (Wobus et al., Exp. Cell. Res., 152:212-219(1984); Matsui et al., Cell, 70:841-847(1992); Resnick et al., Nature, 359:550-551(1992); Shim et al., Biol. Reprod., 57:1089-1095(1997); Piedrahita et al., Biol. Reprod., 58:1321-1329(198)). Almost all of the chicken EG cell colonies were uniformly round and were not tightly bound to the feeder layer. The morphology of the colonies

was multilayered and the boundaries thereof were well-delineated. The chicken EG cell was composed of a large nucleus and a relatively small amount of cytoplasm, while its nucleolous was not prominent. In contrast to mouse ES or EG cells, chicken EG cells did not pack strongly together and therefore it was easy to discern the individual component cells (Fig. 1). Fig. 1 is a photograph under a stereomicroscope showing chicken EG cell colonies after 3 passages on CEF as a feeder cell.

**[EFFECTS OF THIS INVENTION]**

As described previously, the present invention provides a process for preparing an avian embryonic germ cell line, comprising subculturing gonadal primordial germ cells isolated from avian embryo in a medium containing a suitable cell growth factor, differentiation inhibitory factor and feeder cells. The embryonic germ cell lines prepared by this invention are useful in researches on germ cell differentiation and genomic imprinting, so that they enable to produce transgenic birds through transfection of foreign gene or gene targeting, finally permitting to provide a bioreactor or novel breed with improved characteristics.